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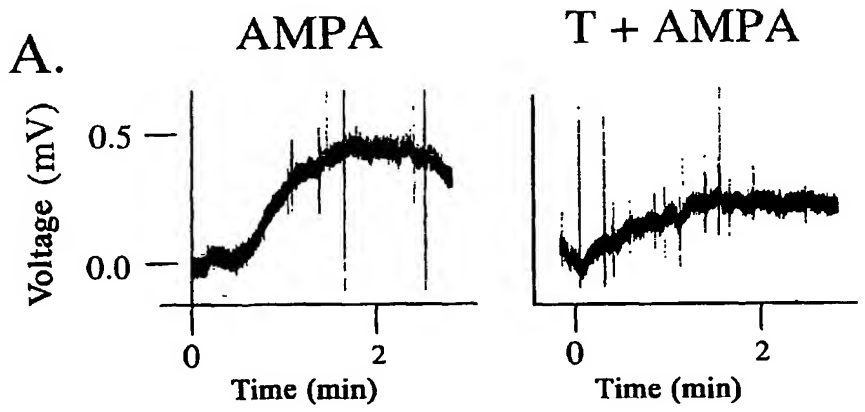
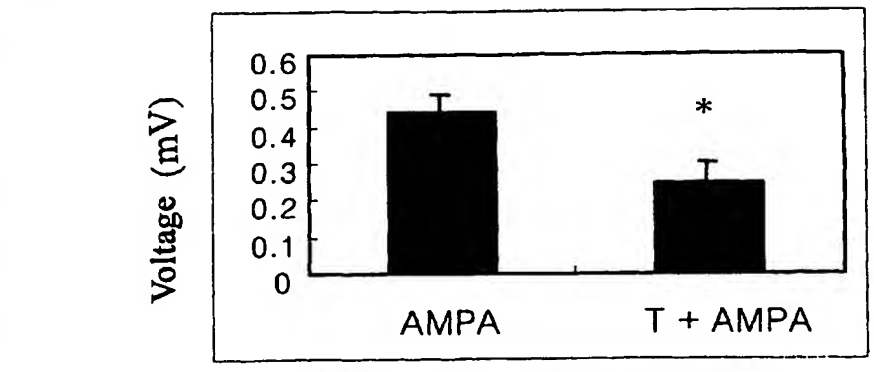
(54) Title: COMPOSITION COMPRISING AN EXTRACT OF LIRIOPSIS TUBER FOR PROTECTING BRAIN CELLS AND IMPROVING MEMORY





(57) Abstract: The present invention relates to a composition comprising an extract of Liriopsis tuber for protecting brain cells or improving memory. The composition of the present invention induces protection of brain cells in men under brain damage by environmental causes such as various stress, drinking and smoking and enhancement of memory, and based on this, can be used as medicines, foodstuffs and beverages which are effective for prevention and treatment of neurodegenerative diseases and for enhancing memory.

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Composition comprising an extract of Liriopsis tuber for protecting brain cells and improving memory

Technical Field

The present invention relates to a composition comprising an extract of Liriopsis tuber for protecting brain cells or improving memory.

Background Art

One of the major factors associated with damage of brain cells is glutamate as an amino acid. Glutamate acts via combining primarily to the four receptors, i.e. NMDA (N-methyl-D-aspartate) receptor, AMPA (L-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptor, Kainate receptor and 1S, 3R-ACPD receptor [Craig CR, Stitzel RE, *Modern Pharmacology with Clinical Applications*, p293-302, 1997]. In the case of ischemia in brain, it causes reduction of oxygen supply to the brain cells, leading to increased anaerobic glycolysis, a decline in the action of ionic pump due to the decreased level of ATP which is an energy source within tissue, increase of the extracellular potassium ion level, resulting in depolarization of neurocellular membrane. In that case, excitatory neurotransmitter is secreted, resulting in brain damage by activation of NMDA, AMPA and Kainate receptors.

Excito-toxicity by excitatory neurotransmitter is known to play a critical role via causing cell stress in the induction of pathological state such as neurodegenerative disorders including Alzheimer's disease, Parkinsonism, stroke and amyotrophic lateral sclerosis [Haloween, B., Reactive oxygen species and the central nervous system. *J. Neurochem.* 59, p1609-1623, 1992; Coyle, J. T. and Puttfarcken, P., Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262, p689-695, 1993; Olanow, C.

W., A radical hypothesis for neurodegeneration. *Trends Neurosci.* 16, p 439-444, 1993]. Neurodegenerative disorders in central nervous system are often accompanied by decline of memory and cognitive function. In particular, dementia is a serious problem in today's aging society, and as the causes, heritage, aging, brain lesion, environmental causes such as smoking and drinking and other complicated factors can be considered. The hippocampus of patients suffering from dementia is heavily damaged and this is closely related to the reduction of acetylcholine levels in the brain. Currently, to raise the acetylcholine level in brain, acetylcholine esterase inhibitors are clinically used in the treatment of Alzheimer's dementia. Besides, lots of studies have been conducted concerning suppression of such brain damage [Gagliardi RJ, Neuroprotection, excitotoxicity and NMDA antagonists, *Arq. Neuro-Psiquiatr.* p58, 2000], and for example, NMDA antagonists, AMPA antagonists, GABA agonists, intracellular calcium reducing agents, nitric oxide inhibitors, free radical scavengers, sodium channel inhibitors, glutamate release inhibitors, growth factors, acidosis, hypothermia and potassium channel activators are under development.

Though dozocyilpin (MK 801), selfotel, cerestat and dextrometorfan have been developed as NMDA antagonists, these drugs, at a low dose, induced changes of cognition, discomfort, nystagmus and hypotension and at a high dose, exhibited mental side effects such as excitation, paranoia and hallucination. In addition, NBQX has been developed as an AMPA antagonist, but industrial applicability as medicine was very low due to serious renal toxicity.

Therefore, development of a brain-protecting agent without toxicity is urgent task in this field.

Recent studies revealed that AMPA receptor might play a key role in the occurrence of Alzheimer's disease in the light of that neuronal cell damage by

activation of AMPA receptor occurs selectively on basal forebrain cholinergic neurons (BFCNs) associated with Alzheimer's disease. This suggests a possibility of developing a therapeutic for Alzheimer's disease based on an AMPA antagonist [Weiss, J. H. *et al.*, Basal forebrain cholinergic neurons are selectively vulnerable to AMPA/kainate receptor-mediated neurotoxicity. *Neuroscience* 60, p 659-664].

Insulin receptors are mainly involved in glucose metabolism in peripheral tissue, while in central nervous system, it plays an important role in regulation of neuroactivity such as memory control rather than glucose metabolism. In fact, insulin receptors are widely distributed in various regions of brain tissue, in particular, dominant in hippocampus. Therefore, hippocampus becomes a primary target with respect to the role of insulin in central nervous system. Lately, many studies showed that insulin and activation of insulin receptor play a major role for memory formation in brain [Park, C. P., Seeley, R. J., Craft, S, and Woods, S. C. (2000), Intracerebroventricular insulin enhances memory in a passive avoidance task. *Physiol. Behav.,* 68, 509-514; Zhao, W., Chen, H., Xu, H., Moore, E., Meiri, N., Quon, M. J., Alkon, D. L., (1999), Brain insulin receptors and spatial memory, *J. Biol. Chem.,* 274, 34893-34902].

Further, ERK (extracellular signal-regulated kinase) I/II are essential signal transduction proteins connecting growth factor-mediated activation of plasma membrane receptor with changes in growth, differentiation and gene expression of cell, and it was reported that activation of ERK I/II in the cellular signal transduction mechanism is important for enhancing memory [Siddhanti et al., *Endocrinology, 136,* 4834-4841 (1995); Hipskind and Bilbe, *Front Biosci., 1,* D804-816 (1998); Thiels, E, Klann, E. Extracellular signal-regulated kinase, synaptic plasticity, and memory, *Rev. Neurosci.* 12, 327-345 (2001); Sweat J. D. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory, *J.*

Neurochem. 76, 1-10, (2001)].

Accordingly, it is considered that materials inducing the activation of insulin receptors and ERK I/II could be used for memory enhancement or dementia medicines in addition to cholinesterase inhibitors.

The inventor of the present invention has continued studies on the substance which can induce brain cells protecting effect and memory-improving effect in men suffering from brain damage by environmental causes such as stress, drinking and smoking, and as a result, discovered that an extract of Liriopsis tuber exhibits a superior effect on protecting brain cells and improving memory, and based on this, completed the present invention..

Therefore, the object of the present invention is to provide a composition comprising an extract of Liriopsis tuber for protecting brain cells or enhancing memory.

Disclosure of Invention

The present invention relates to a composition comprising an extract of Liriopsis tuber for protecting brain cells or improving memory.

The composition of the present invention for protecting brain cells or improving memory, includes a Liriopsis tuber extract by 0.5 to 50% by weight based on the total weight of the composition.

Liriopsis tuber is a perennial herb classified into Liliaceae, and includes *Liriope platyphylla* Wang *et* Tang, *Ophiopogon japonicus* Ker-Gawl., O. stolonifer Levl. et Vant., Mondo japonicum (L.f.) Farwell and *Liriope spicata* (Thunb.) Lour., and swelling part of root is used for medicinal purposes. It contains components such as glucose, fructose, sucrose, ophiopogonone A and B, methylopiogonone A and B, ophiopogonanone A, methylopiopogonanone A and B, homoisoflavonoid I~V, borneol

glycoside, β-sitosterol, stigmasterol, β-sitosterol glycoside, oligosaccharides, polysaccharides, 3-O-α-L-rhamnopyranosyl(1-2)-β-D-glucopyranosylophiogenin, 5,7-dihydroxy-6-formyl-8-methyl-3-(3,4-methylenedioxybenzyl)chroman-4-one, 6-aldehydo-isoophiopogonanone A, 6-aldehydo-isoophiopogonone A, 6-aldehydo-isoophiopogonone B, 6-aldehydo-ophiopogonone A, 7-O-α-L-arabinofuranosyl(1-6)-β-D-glucopyranosylborneol, 7-O-β-D-glucopyranosyl-borneol, azetidine-2-carboxylic acid, daucosterol, methylophiopogonanone A, methylophiopogonanone B, mono-O-acetylophiopogonin D, ophiopogon C, ophiopogon amide VI, ophiopogon homoisoflavonoid I, ophiopogon homoisoflavonoid II, ophiopogon homoisoflavonoid III, ophiopogon homoisoflavonoid IV, ophiopogon homoisoflavonoid V, ophiopogonanone A, ophiopogonin A, ophiopogonin B', ophiopogonin B, ophiopogonin C', ophiopogonin D, ophiopogonin D', tulipanin, vicenin 2, 25(S)-ruscogenin-1-O-α-L-rhamnopyranosyl(1-2)-β-D-fucopyranoside, 25(S)-ruscogenin-1-O-β-D-xylopyranosyl(1-3)-β-D-fucopyranoside, aster saponin Hb methyl ester, Lm-2, Lm-3, Ls-2, Ls-3, Ls-4, Ls-5, Ls-6, Ls-7, ruscogenin-1-sulfate-3-O-α-L-rhamnopyranoside, 1-sulfate-3-O-α-L-rhamnopyranosyl-ruscogenin, ruscogenin-3-O-α-L-rhamnopyranoside and ruscogenin-3-O-β-D-glucopyranosyl(1-3)-α-L-rhamnopyranoside, and used in oriental medicine for suppression of cough, expectoration, nutrition, sthenia, diuresis, suppression of thirst, blood glucose regulation, xerostomia and constipation [Illustrated Dictionary of Folk Medicine by Bosup Chung and Minkyo Shin, Younglim company p177-178, 1998 and New Oriental Medicine Index Traditional Medicine Database (TradMed), Natural Products Research Institute of Seoul National University, revised ed., 1999].

Nevertheless, there has not yet been a report on that a Liriopsis tuber extract has an effect on protecting brain cells and improving memory.

The Liriopsis tuber extract according to the present invention can be prepared by the following method.

Extraction method 1: the Liriopsis tuber extract can be obtained by extracting with a solvent selected from the group consisting of C $_{1-4}$ lower alcohols or a mixture of said lower alcohols with water, acetone, chloroform, methylene chloride, ether and ethyl acetate, preferably, methanol or a mixed solvent of methanol and water in ratio of 1:0.2-1.5. The reaction temperature is 5 to 80°C, preferably 30 to 55°C, and reaction time is 15 min to 48 hr, preferably 30 min to 12 hr.

The solvent soluble fraction thus obtained contains a large amount of terpenoids and phenolic substances.

Extraction method 2: The solvent soluble fraction obtained as described in said Extraction method 1 was dissolved in a mixture of C $_{1-4}$ lower alcohol and water and pH was adjusted with an acid to a range of 2-4 and then extraction was further conducted using an equal amount of chloroform to obtain a chloroform soluble fraction.

Extraction method 3: The chloroform insoluble fraction of the fractions obtained according to said Extraction method 2 was treated with ammonium hydroxide to adjust pH to 9-12 and extracted with an equal amount of a mixture of chloroform-methanol, and the fraction insoluble in the chloroform-methanol mixture was further extracted with methanol to obtain a methanol soluble fraction and a methanol insoluble aqueous fraction.

At this time, a mixing ratio of the chloroform-methanol mixed solvent is preferred to be 1:0.1~1. Of the chloroform insoluble fraction, the fraction dissolved upon extraction with the mixed solvent (chloroform-methanol) contains the majority of alkaloids, and the methanol-soluble fraction of the solvent (chloroform:methanol)-insoluble fraction includes quaternary alkaloids and N-oxides.

In addition, the Liriopsis tuber extract of the present invention can undergo additional fractionation process by conventional method (Harborne J. B. Phytochemical methods: *A guide to modern techniques of plant analysis,* 3rd Ed., pp 6-7, 1998).

The composition of the present invention comprising an extract of Liriopsis tuber can further include at least one component selected from the group consisting of pharmaceutically acceptable carriers and additives according to conventional method.

The carrier that can be included in the composition comprising the extract of Liriopsis tuber of the present invention also includes substances commonly called excipients or diluents, and for example, at least one component selected from the group consisting of lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starch, isomerized sugar, sugar, acacia gum, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoate, propylhydroxybenzoate, paraoxybenzoate, methylparaoxybenzoate, paraoxypropylbenzoate, talc, magnesium stearate and mineral oil, can be used.

In addition, as the additives that can be included in the composition comprising the extract of Liriopsis tuber of the present invention, at least one component selected from the group consisting of natural carbohydrates, flavors, nutrients, vitamins, mineral (electrolytes), seasonings (synthetic, natural seasonings), coloring agents, fillers (cheese, chocolate, etc.), pectic acid and its salt, alginic acid and its salt, organic acids, protective colloidal thickeners, pH regulating agents, stabilizers, preservatives, antioxidants, glycerin, alcohols, carbonizing agents and sarcocarp, can be used.

The composition comprising the extract of Liriopsis tuber of the present invention can be used via formulating into oral administration such as powders, tablets, capsules, suspensions, emulsions, syrups and aerosols; topical applications;

suppositories or sterile injections.

Though the amount of the Liriopsis tuber extract used differs depending on the age, sex, body weight of patient, daily amount of 0.1 to 500mg/kg can be administered by dividing into one to several times. Further, the administered amount of the extract of Liriopsis tuber and its fractions can be controlled according to administration route, seriousness of disease, sex, weight and age, and said amount never limits the scope of the invention in any way. The extract of Liriopsis tuber itself according to the present invention is almost free of toxicity and adverse effect so that it can be safely used even in the case of prolonged use for the prevention purpose.

The extract of Liriopsis tuber of the present invention can also be used, together with sitologically acceptable additives, for various foodstuffs, beverages, gums, teas, vitamin complexes and health foods or beverages.

In the case of a foodstuff containing the extract of Liriopsis tuber of the present invention, the content of the Liriopsis tuber extract is 0.1 to 15% by weight, preferably 1 to 10% by weight based on the total weight of the foodstuff.

In addition, in the case of a beverage containing the extract of Liriopsis tuber of the present invention, the content of the extract of Liriopsis tuber is 1 to 30g, preferably 3 to 10g per 100ml of the beverage.

Also, as the sitologically acceptable additives that can be included in the foodstuff or the beverage according to the present invention, at least one component selected from the group consisting of natural carbohydrates, flavors, nutrients, vitamins, mineral (electrolytes), seasonings (synthetic or natural seasonings), coloring agents, fillers (cheese, chocolate, etc.), pectic acid and its salt, alginic acid and its salt, organic acids, protective colloidal thickeners, pH regulating agents, stabilizers, preservatives, antioxidants, glycerin, alcohols, carbonizing agents and sarcocarp, can be used.

It is preferred for said additives to be contained in a range of 0.01 to 25 parts by weight based on 100 parts by weight of the foodstuff or beverage composition.

Additionally, as natural carbohydrates, monosaccharides such as glucose and fructose; disaccharides such as maltose and sucrose; polysaccharides such as dextrin and cyclodextrin; and sugar alcohol such as xylitol, sorbitol and erythritol can be used, and it can be generally used in an amount of about 1 to 20g, preferably, about 5 to 12g per 100ml of the beverage composition.

As flavors, natural flavors such as taumatin and stevia extract (e.g. rebaudioside A, glycyrrhizin etc.); and synthetic flavors such as saccharin and aspartam can be used.

The beverage composition of the present invention has no other limitation on liquid components except containing said extract of Liriopsis tuber as an essential component at the indicated ratio.

Brief Description of Drawings

Fig. 1 represents an inhibiting effect of a Liriopsis tuber extract (fraction T) against the depolarization of nerve cell by AMPA. The value indicated (Fig. 1B) mean ± standard deviation (n=5), and significance to the control group is *: $P<0.05$.

Fig. 2 represents an inhibiting effect of a Liriopsis tuber extracts (fractions A, C, CM and M) against the depolarization of nerve cell by AMPA. The value indicated mean ± standard deviation (n=5), and significance to the control group is *: $P<0.05$ and **: $P<0.01$.

Fig. 3 shows a memory-enhancing effect of a Liriopsis tuber extract (fraction T). The value indicated mean ± standard deviation (n=8), and significance to the control group is *: $P<0.05$.

Fig. 4 shows a memory-enhancing effect of Liriopsis tuber extracts (fractions T,

A, C, CM and M). The value indicated mean ± standard deviation (n=7), and significance to the control group is *: P<0.05.

Fig. 5 demonstrates an inhibiting effect of Liriopsis tuber extracts (fractions T, A, C and M) against acetylcholine esterase. The value indicated mean ± standard deviation (n=6), and significance to the control group is ***: P<0.001.

Fig. 6 represents an enhancing effect of Liriopsis tuber extracts (fractions T, A, C and M) on ERK I and ERK II activity.

Fig. 7 represents an increasing effect of Liriopsis tuber extracts (fractions T, A, C and M) on insulin receptor activity.

Best Mode for Carrying Out the Invention

The present invention is described in more detail with the following Examples, yet they do not limit the scope of the present invention.

Example 1: Preparation of an extract of Liriopsis tuber

Liriopsis tuber 250g was cut into small pieces and subjected to three times of extraction, each time, with 70% methanol (750ml) using Soxhlet apparatus. The extract was filtered, subjected to concentration at a reduced pressure using a rotary evaporator (EYELA N-N series) and subjected to lyophilization to obtain a crude methanol extract (fraction T).

For further fractionation with other organic solvent, said lyophilized methanol extract 10g was dissolved in a mixture of methanol and water (4:1) 200ml, adjusted with 2M sulfuric acid to pH 3, extracted successively three times, each time, with an equal amount of chloroform, subjected to concentration at a reduced pressure and lyophilization to obtain a chloroform soluble fraction (fraction C), 0.12g, and the

aqueous layer was adjusted with ammonium hydroxide to pH 10, extracted two times, each time, with an equal amount of a mixture of chloroform and methanol (3:1). The chloroform-methanol (3:1) layer was subjected to concentration at a reduced pressure and lyophilization to obtain a chloroform-methanol soluble fraction (fraction CM), 0.09g. The aqueous layer was extracted three times, each time with an equal amount of methanol, subjected to concentration at a reduced pressure and lyophilization to obtain a methanol soluble fraction (fraction M), 2.94g and a water soluble fraction (fraction A), 2.75g, respectively, and the fractions was used as a sample in the following activity assay.

Experimental Example 1: Grease Gap assay

1) Experimental method

Wedges were prepared from cerebral cortex in white rats and placed on a two compartments brain bath, and test was performed [Harrison NL, Simmonds, MA, Quantitative studies on some antagonists of N-methyl D-aspartate in slices of rat cerebral cortex, *Br. J. Pharmacol.* 84, p381-391, 1985]. Brain was quickly taken out and 2-3mm of the front part was removed using a brain tissue slicer and then the remaining part was subjected to vertical cut to prepare a coronal section of 500-600μm thickness and rapidly put into an oxygenated Krebs medium, and divided into two parts against median line to prepare wedges in which dorsal surface containing cerebral cortex and corpus callosum was approximately 1.5mm wide and ventral surface was approximately 1mm wide. After being left on the oxygenated Krebs medium for 2 hr at room temperature, the wedges were placed through a greased (high vacuum silicone grease) slot in a two compartments brain chamber. The two compartments were perfused with Krebs medium at a speed of 2ml/min. The extracts of Liriopsis tuber

(fractions T, A, C, CM and M) were perfused to the cortical end of the preparation at a concentration of 10μg/ml for 10 min and excitatory amino acid, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) (40μM) was then applied by 2 min superfusion, and d.c. potential between the two compartments was measured with Ag/AgCl electrode, amplified by amplifier, and determined with McLab Data Acquisition System. Control experiments were also performed with AMPA only.

2) Experimental result

Induction of depolarization of nerve cell by AMPA is considered as a barometer of stimulus by nerve cell lesion. As a result of experiment, as can be seen from Figs. 1A and 1B, application of AMPA (40μM) induced depolarization of 0.44mV, while application of AMPA after pretreatment with a Liriopsis tuber extract (fraction T) (10μg/ml) markedly reduced the level of depolarization to 0.24mV. In particular, pretreatments with other fractions of Liriopsis tuber extracts (fractions A, C and M) demonstrated an inhibiting effect against depolarization by AMPA, i.e. 66%, 48% and 63%, respectively (Fig. 2).

Therefore, it can be seen that the nerve protecting effect is induced by various components in the extracts of Liriopsis tuber.

Experimental Example 2: $NaNO_2$ memory test

It is known that oxygen metabolism deficiency of brain by $NaNO_2$ is closely associated with cholinergic neurotransmission related with memory and learning [Schindler et al., Nootropic drugs: Animal models for studying effects on cognition. *Drug Develop Res* 4: p567-576, 1984], and in particular, an oxidative metabolism disorder in brain by $NaNO_2$ is intimately connected with memory disturbance owing to cholinergic blockade. Therefore, if a delay is observed in the time for $NaNO_2$ –

mediated death induction upon drug treatment, it can be considered as one of indications reflecting memory-improving effect of the drug.

1) Experimental method

An extract of Liriopsis tuber (fraction T) was administered (10mg/kg) via P.O. to male mice (20g) and after 60 min, $NaNO_2$ (250mg/kg) was administered via s.c. Time period until breath stops was measured, and breath-duration time was compared with that of control group to evaluate a memory-improving effect.

2) Experimental result

As can be seen from Fig. 3, pretreatment with the Liriopsis tuber extract (fraction T) (10mg/kg, P.O.) induced 45% increase in the time for death induction due to the brain metabolism disorder by $NaNO_2$, revealing a memory improving effect of the Liriopsis tuber extract.

Experimental Example 3: Passive Avoidance test

1) Experimental method

Male mice (20g) were administered with Liriopsis tuber extracts (fractions T, A, C, CM and M) via P.O. route for three days (10mg/kg per day), and a passive avoidance test was performed using Gemini Avoidance System (San Diego Instruments, USA). The experiment was carried out basically according to Kumar et al. method with some modifications [Kumar, V., Singh, P.N., Muruganandan, A. V., Bhattacharya. Effect of Indian Hypericum perforatum Linn on animal models of cognitive dysfunction, *J. Ethnopharmacology* 72, p119-128, 2000].

In the case of training experiment on the first day, the mice were put into a light box, subjected to acclimation for 300 sec and then allowed to move into a dark box by making the door to be opened automatically. Upon moving into the dark box, electric

stimulus of 0.3mA was given for 1 sec. Scopolamine was administered (1mg/kg, i.p.) immediately after termination of the training session. In the case of test experiment conducted after 24 hr, the mice were subjected to acclimation for 300 sec in the light box, the door was opened and the mice were allowed to move into the dark box. Time for moving into the dark box was measured. On the second day, electric stimulus was not given. If mouse stayed without moving into the dark box for 500 sec, maximum score, 500 sec was given.

2) Experimental result

In the experiment on the first day, there was no significant difference among the groups. In the test experiment on the second day, it has been found that mice with scopolamine-induced dementia have decreased memory by 83% to the control group. However, the mice administered with Liriopsis tuber extracts (fractions T, A, C and M) for 3 days, restored memory up to 33%, 32%, 45% and 158%, respectively, against memory disorder due to scopolamine.

Experimental Example 4: *Ex vivo* cholinesterase assay

1) Experimental method

Male SD rats were orally administered with an extract of Liriopsis tuber (10mg/kg) and after 60 min, brain was taken out and then hippocampus was separated, resuspended with an isolation buffer containing 50mM Tris HCl, 1mM EDTA, 1mM EGTA, 150mM NaCl, 1% Triton X-100, 0.5mM PMSF, 1mM Na_3VO_4, 1μg/ml leupeptin and 1μg/ml aprotinin, and homogenized with a Potter-Elvehjem homogenizer. The insoluble material was removed by centrifugation for 20min (10,000 x g) at 4℃. Activity of cholinesterase was determined by Ellman et al. method [Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M. A new and rapid colorimetric

determination of acetylcholinesterase activity, *Biochem. Pharmacol.* 7, 88-95, 1961]. Buffer I (100mM phosphate, pH 8.0) 3ml, 75mM acetylthiocholine iodide 0.2ml and buffered Ellmans reagent (DTNB 10mM, $NaHCO_3$ 15mM) 0.1ml were mixed and reacted at 25°C for 10 min. To this reaction mixture, hippocampus lysate 20μl was added and absorbance was determined by 30 sec interval. Percentage inhibition was calculated via comparing with the control group.

2) Experimental result

The administration of extracts of Liriopsis tuber (fractions T, A, C and M) inhibited cholinesterase activity by 56%, 64%, 56% and 44%, respectively (Fig. 5).

Experimental Example 5: Effect on ERK I/II activity

1) Experimental method

Effect of extracts of Liriopsis tuber on the activity of ERK (Extracellular signal-regulated kinase) I/II was determined as follows.

Each fractions of Liriopsis tuber extracts was orally administered (10mg/kg), hippocampus was separated after 1 hr and put into the isolation buffer as prepared in the Experimental Example 4 and homogenized with a Potter-Elvehjem homogenizer, and ERK I/II activity was determined based on SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western blotting as described below.

For the PAGE, 30$\mu\ell$ samples containing an equal amount of protein were applied. Five folds Laemmli's sample buffer was added, boiled in water for 5 min and sample thus obtained was loaded and subjected to electrophoresis at 100V. SDS-PAGE condition: 7.5% resolving gel. After carrying out SDS-PAGE, proteins were allowed to transfer to nitrocellulose membrane for 1 hr at 100V using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The nitrocellulose membrane was soaked in

blocking solution prepared by dissolving 5% skim milk in PBS solution containing 0.1% Tween 20 (PBS-T), and incubated overnight in 4°C refrigerator. Solution of primary antibody (anti ERK I/II Ab and anti-phospho ERK I/II Ab; New England Biolab, USA) diluted with PBS-T to 1:1000 was added to the nitrocellulose membrane and reacted for 1 hr. The nitrocellulose membrane was washed with PBS-T once for 15 min, three times for 5 min, and solution of secondary antibody (horseradish peroxidase-linked goat anti-rabbit IgG (Pierce)) diluted with PBS-T to 1:1000 was added and reacted for 40 min. ERK II (42 kDa) protein band was identified by enhanced chemiluminescence (ECL, Pierce) (Harlow E. and Lane D., *Antibodies: A laboratory manual, 726*, 1988).

2) Experimental result

As a result, the amount of ERK I/II activated via phosphorylation (phospho-ERK I/II) was seen to be remarkably increased upon administration of Liriopsis tuber extracts (fractions T, A, C and M) when compared to the control group (Fig. 6A). On the other hand, protein content of ERK I/II was nearly equal in both control group and administered groups (Fig. 6B). Based on this result, it can be confirmed that the extracts of Liriopsis tuber (fractions T, A, C and M) induce memory-improving action via activating ERK I/II of hippocampus in rat.

Experimental Example 6: Effect on the activity of insulin receptor

Activation of insulin receptor plays an important role on memory formation. As the activation of insulin receptor is induced via phosphorylation of tyrosine residue in β subunit thereof, the effect of a Liriopsis tuber extract on the activation of insulin receptor was analyzed as follows.

1) Experimental method

Each fraction of the extracts of Liriopsis tuber was orally administered (10 mg/kg concentration) to rats and after 1 hr, hippocampus was separated, placed in 4 folds of the isolation buffer as prepared in Experimental Example 4 to the weight of hippocampus and homogenized using a Potter-Elvehjem homogenizer. Then, activity of insulin receptor was determined as described below by immunoprecipitation reaction and SDS-PAGE electrophoresis, and Western blotting.

Homogenized hippocampus lysate 100μl was mixed with buffer (0.5M NaCl, 1% NP-40, 10% deoxycholate, 0.1% SDS) 100μl, reacted at 4° C for 1 hr, and the solubilized proteins were isolated by centrifugation. To the supernatant, insulin receptor antibody (Transduction laboratories) (5μl) was added. The reaction mixture was allowed to rotate using a rotor for 1 hr and protein A Sepharose (20μl) was added and reacted at 4 °C for 1 hr using the rotor. The immune complex was precipitated by centrifugation. The pellets were washed with washing buffers A (0.01M Tris, pH 7.4, 1M NaCl, 1% Nonidet P-40), B (0.01M Tris, pH 7.4, 0.1M NaCl, 0.01M EDTA, 1% Nonidet P-40, 0.3% SDS) and C (0.01M Tris, pH 7.4, and 1% Nonidet P-40), sequentially. The final pellets were solubilized with Laemmli's sample buffer containing 100mM dithiothreitol, boiled for 5 min, centrifuged in a microcentrifuge, and the supernatant was subjected to SDS-PAGE: 7.5% resolving gel. After electrophoresis, Western blotting was conducted as described in Experimental Example 3, and phosphorylation of tyrosine residue on insulin receptor β subunit was observed using phosphotyrosine Ab (Transduction laboratories) as an antibody.

2) Experimental result

As it can be seen from Fig. 7, the fraction T of the Liriopsis tuber extract noticeably activated insulin receptor and fraction C as well exhibited remarkable effect on activating insulin receptor compared to the control group. Accordingly, it can be

concluded that activation of insulin receptor plays a critical role in the memory-improving effect by fractions T and C confirmed in said Experimental example 3.

Formulation Example 1. Tablets

Tablets of the following components were formulated according to conventional manufacturing method for tablets.

1-1. Tablet composition

Methanol extract of Liriopsis tuber	500.0mg
Lactose	500.0mg
Talc	5.0mg
Magnesium stearate	1.0mg

1-2. Tablet composition

Chloroform fraction of methanol extract of Liriopsis tuber	50.0mg
Lactose	50.0mg
Talc	0.5mg
Magnesium stearate	0.1mg

1-3. Tablet composition

Methanol fraction of methanol extract of Liriopsis tuber	50.0mg
Lactose	50.0mg
Talc	0.5mg
Magnesium stearate	0.1mg

1-4. Tablet composition

Methanol-insoluble fraction of methanol extract of Liriopsis tuber	50.0mg
Lactose	50.0mg
Talc	0.5mg
Magnesium stearate	0.1mg

Formulation Example 2. Capsules

Based on the following composition, capsules were prepared by the following process. The extract of Liriopsis tuber was screened, mixed with excipient, filled into gelatin capsule to prepare capsules.

2-1. Capsule composition

Methanol extract of Liriopsis tuber	500.0mg
Starch 1500	10.0mg
Magnesium stearate BP	100.0mg

2-2. Capsule composition

Chloroform fraction of methanol extract of Liriopsis tuber	50.0mg
Starch 1500	1.0mg
Magnesium stearate BP	10.0mg

2-3. Capsule composition

Methanol fraction of methanol extract of Liriopsis tuber	50.0mg
Starch 1500	1.0mg
Magnesium stearate BP	10.0mg

2-4. Capsule composition

Methanol insoluble fraction of methanol extract of Liriopsis tuber	50.0mg
Starch 1500	1.0mg
Magnesium stearate BP	10.0mg

Formulation Example 3. Syrups

Based on the following composition, syrups were prepared as follows. Sugar was dissolved in purified water and then paraoxybenzoate, paraoxypropylbenzoate and Liriopsis tuber extract were added, dissolved at 60°C, cooled and purified water was added to 150ml.

3-1. Syrup composition

Methanol extract of Liriopsis tuber	5.0g
Sugar	95.1g
Paraoxybenzoate	80.0mg
Paraoxypropylbenzoate	16.0mg
Purified water	to 150ml

3-2. Syrup composition

Chloroform fraction of methanol extract of Liriopsis tuber	50.0mg
Sugar	95.1g
Paraoxybenzoate	80.0mg
Paraoxypropylbenzoate	16.0mg
Purified water	to 150ml

3-3. Syrup composition

Methanol fraction of methanol extract of Liriopsis tuber	50.0mg
Sugar	95.1g
Paraoxybenzoate	80.0mg
Paraoxypropylbenzoate	16.0mg
Purified water	to 150ml

3-4. Syrup composition

Methanol insoluble fraction of methanol extract of Liriopsis tuber	50.0mg
Sugar	95.1g
Paraoxybenzoate	80.0mg
Paraoxypropylbenzoate	16.0mg
Purified water	to 150ml

Formulation Example 4. Solutions

The following components were formulated by conventional process, and filled into brown bottles to prepare solutions.

4-1. Solution composition

Methanol extract of Liriopsis tuber	500.0mg
Isomerized sugar	20.0g
Antioxidant	5.0mg
Methyl paraoxybenzoate	2.0mg
Purified water	to 100.0ml

4-2. Solution composition

Chloroform fraction of methanol extract of Liriopsis tuber	500.0mg
Isomerized sugar	20.0g
Antioxidant	5.0mg
Methyl paraoxybenzoate	2.0mg
Purified water	to 100.0ml

4-3. Solution composition

Methanol fraction of methanol extract of Liriopsis tuber	500.0mg
Isomerized sugar	20.0g
Antioxidant	5.0mg
Methyl paraoxybenzoate	2.0mg
Purified water	to 100.0ml

4-4. Solution composition

Methanol insoluble fraction of methanol extract of Liriopsis tuber	500.0mg
Isomerized sugar	20.0g
Antioxidant	5.0mg
Methyl paraoxybenzoate	2.0mg
Purified water	to 100.0ml

Formulation Example 5. Powders

The following components were mixed, filled in bag and sealed to prepare powders by conventional method for powders.

5-1. Powder composition

Methanol extract of Liriopsis tuber	50.0mg
Lactose	100.0mg
Talc	5.0mg

5-2. Powder composition

Chloroform fraction of methanol extract of Liriopsis tuber	50.0mg
Lactose	100.0mg
Talc	5.0mg

5-3. Powder composition

Methanol fraction of methanol extract of Liriopsis tuber	50.0mg
Lactose	100.0mg
Talc	5.0mg

5-4. Powder composition

Methanol insoluble fraction of methanol extract of Liriopsis tuber	50.0mg
Lactose	100.0mg
Talc	5.0mg

Formulation Example 6. Injections

The following components were filled in 2.0ml amples, subjected to sterilization to prepare injections by conventional process for injections.

6-1. Injection composition

Methanol extract of Liriopsis tuber	50.0mg
Antioxidant	1.0mg
Tween 80	1.0mg
Distilled water for injection	to 2.0ml

6-2. Injection composition

Chloroform fraction of methanol extract of Liriopsis tuber	50.0mg
Antioxidant	1.0mg
Tween 80	1.0mg
Distilled water for injection	to 2.0ml

6-3. Injection composition

Methanol fraction of methanol extract of Liriopsis tuber	50.0mg
Antioxidant	1.0mg
Tween 80	1.0mg
Distilled water for injection	to 2.0ml

6-4. Injection composition

Methanol insoluble fraction of methanol extract of Liriopsis tuber	50.0mg
Antioxidant	1.0mg
Tween 80	1.0mg
Distilled water for injection	to 2.0ml

Formulation Example 7. Preparation of Sunsik

Brown rice, barley, glutinous rice and Job's tear were gelatinized, dried, parched and ground to 60 mesh powder according to conventional method. Black bean, black sesame and Perilla japonica as well were boiled, dried, parched and ground to 60 mesh powder by conventional method. Grains, seed-fruits and dried extract of Liriopsis tuber prepared as described above were combined by the following ratio to prepare granules.

7-1. Preparation Example of Sunsik

Grains: brown rice 30w/w%, Job's tear 15%, barley 20w/w% and glutinous rice 9%

Seed-fruits: Perilla japonica 7w/w%, black bean 8w/w% and black sesame 7w/w%

Dried powder of methanol extract of Liriopsis tuber: 3w/w%, Ganoderma Lucidum (FR) karst 0.5w/w% and Rehmannia glutinosa 0.5w/w%

7-2. Preparation Example of Sunsik

Grains: brown rice 30w/w%, Job's tear 15%, barley 20w/w% and glutinous rice 9%

Seed-fruits: Perilla japonica 7w/w%, black bean 8w/w% and black sesame 7w/w%

Dried powder of chloroform fraction in methanol extract of Liriopsis tuber: 3w/w%, Ganoderma Lucidum (FR) karst 0.5w/w% and Rehmannia glutinosa 0.5w/w%

7-3.Preparation Example of Sunsik

Grains: brown rice 30w/w%, Job's tear 15%, barley 20w/w% and glutinous rice 9%

Seed-fruits: Perilla japonica 7w/w%, black bean 8w/w% and black sesame 7w/w%

Dried powder of methanol fraction in methanol extract of Liriopsis tuber: 3w/w%, Ganoderma Lucidum (FR) karst 0.5w/w% and Rehmannia glutinosa 0.5w/w%

7-4.Preparation Example of Sunsik

Grains: brown rice 30w/w%, Job's tear 15%, barley 20w/w% and glutinous rice 9%

Seed-fruits: Perilla japonica 7w/w%, black bean 8w/w% and black sesame 7w/w%

Dried powder of methanol-insoluble fraction from methanol extract of Liriopsis tuber: 3w/w%, Ganoderma Lucidum (FR) karst 0.5w/w% and Rehmannia glutinosa 0.5w/w%

Industrial Applicability

The composition comprising an extract of Liriopsis tuber of the present invention exhibits effects of preventing and treating neurodegenerative diseases caused by brain cell damage and of improving memory and accordingly, it can be used for protecting brain cells in persons under the risk of brain damage by various environmental stress and for improving memory in persons suffering from memory decline including dementia.

CLAIMS

1. A composition comprising an extract of Liriopsis tuber for protecting brain cells or improving memory.

2. The composition of claim 1, wherein the content of the extract of Liriopsis tuber is 0.5-50% by weight based on the total weight of the composition.

3. The composition of claim 1, wherein the extract of Liriopsis tuber is obtained by extracting with a solvent selected from the group consisting of C_{1-4} lower alcohols or a mixture of said lower alcohols and water, acetone, chloroform, methylene chloride, ether and ethyl acetate.

4. The composition of claim 1, wherein the extract of Liriopsis tuber is obtained by dissolving the solvent soluble fraction obtained as described in claim 3 in a mixed solvent of C_{1-4} lower alcohol and water, adjusting pH value with an acid to a range of 2-4, and further fractionating via extraction with an equal amount of chloroform.

5. The composition of claim 1, wherein the extract of Liriopsis tuber is obtained by dissolving the solvent soluble fraction obtained as described in claim 3 in a mixed solvent of C_{1-4} lower alcohol and water, adjusting pH value with an acid to a range of 2-4, further extracting with an equal amount of chloroform, adjusting pH value of the chloroform insoluble fraction with ammonium hydroxide to a range of 9-12, extracting the chloroform insoluble fraction with an equal amount of chloroform-methanol mixture, further extracting the chloroform-methanol insoluble fraction with methanol,

fractionating, thereby obtaining the extract of Liriopsis tuber from the methanol soluble fraction.

6. The composition of claim 1, wherein the extract of Liriopsis tuber is obtained by dissolving the solvent soluble fraction obtained as described in claim 3 in a mixed solvent of C_{1-4} lower alcohol and water, adjusting pH value with an acid to a range of 2-4, further extracting with an equal amount of chloroform, adjusting pH value of the chloroform insoluble fraction with ammonium hydroxide to a range of 9-12, extracting the chloroform insoluble fraction with an equal amount of chloroform-methanol mixture, further extracting the chloroform-methanol insoluble fraction with methanol, fractionating, thereby obtaining the extract of Liriopsis tuber from the methanol insoluble fraction.

7. The composition of claim 1, wherein said composition further comprises at least one component selected from the group consisting of pharmaceutically acceptable carriers and additives.

8. The composition of claim 1, wherein the composition is formulated into oral administration, topical applications, suppositories or sterile injections.

9. Foodstuff comprising the composition according to claim 1 and a sitologically acceptable additive.

10. The foodstuff of claim 9, wherein the content of the extract of Liriopsis tuber is 0.1 to 15% by weight based on the total weight of foodstuff.

11. The foodstuff of claim 9, wherein said sitologically acceptable additive is at least one component selected from the group consisting of natural carbohydrates, flavors, nutrients, vitamins, minerals, seasonings, coloring agents, fillers, pectic acid and its salt, alginic acid and its salt, organic acids, protective colloidal thickeners, pH regulating agents, stabilizers, preservatives, antioxidants, glycerin, alcohols, carbonizing agents and sarcocarp.

12. A beverage comprising the composition according to claim 1 and a sitologically acceptable additive.

13. The beverage of claim 12, wherein the content of the extract of Liriopsis tuber is 1-30g per 100ml of the beverage.

14. The beverage of claim 12, wherein said sitologically acceptable additive is at least one component selected from the group consisting of natural carbohydrates, flavors, nutrients, vitamins, minerals, seasonings, coloring agents, fillers, pectic acid and its salt, alginic acid and its salt, organic acids, protective colloidal thickeners, pH regulating agents, stabilizers, preservatives, antioxidants, glycerin, alcohols, carbonizing agents and sarcocarp.

15. A method for protecting brain cells against damage caused by excitatory amino acids and oxidative stress in a mammal comprising administering to said mammal a therapeutic amount of an extract of Liriopsis tuber.

16. The method of claim 15, wherein said extract of Liriopsis tuber is administered in an amount of from 0.1mg/kg to 500mg/kg.

17. The method of claim 16, wherein said extract is administered on a daily basis.

18. The method of claim 15, wherein said extract is administered to said mammal via a route selected from the group consisting of oral administration, topical application, sterile injection, inhalation and rectal administration.

19. The method of claim 15, wherein said extract is concurrently administered with a pharmaceutically acceptable carrier, excipient or diluent.

20. The method of claim 15, wherein said administration comprises combining said extract with a beverage, and then orally administering said beverage.

21. The method of claim 15, wherein said administration comprises combining said extract with a foodstuff, and then orally administering said foodstuff.

22. A method for inhibiting AMPA-induced depolarization of a neuronal cell of a mammal comprising administering to said mammal a therapeutic amount of an extract of Liriopsis tuber.

23. The method of claim 22, wherein said extract of Liriopsis tuber is administered in an amount of from 0.1mg/kg to 500mg/kg.

24. The method of claim 23, wherein said extract is administered on a daily basis.

25. The method of claim 22, wherein said extract is administered via a route selected from the group consisting of oral administration, topical application, sterile injection, inhalation and rectal administration.

26. The method of claim 22, wherein said extract is concurrently administered with a pharmaceutically acceptable carrier, excipient or diluent.

27. The method of claim 22, wherein said administration comprises combining said extract with a beverage, and then orally administering said beverage.

28. The method of claim 22, wherein said administration comprises combining said extract with a foodstuff, and then orally administering said foodstuff.

29. A method of facilitating tyrosine phosphorylation of a hippocampal protein of a mammal comprising administering to said mammal a therapeutic amount of an extract of Liliopsis tuber.

30. The method of claim 29, wherein said extract of Liriopsis tuber is administered in an amount of from 0.1mg/kg to 500mg/kg.

31. The method of claim 30, wherein said extract is administered on a daily

basis.

32. The method of claim 29, wherein said extract is administered via a route selected from the group consisting of oral administration, topical application, sterile injection, inhalation and rectal administration.

33. The method of claim 29, wherein said extract is concurrently administered with a pharmaceutically acceptable carrier, excipient or diluent.

34. The method of claim 29, wherein said administration comprises combining said extract with a beverage, and then orally administering said beverage.

35. The method of claim 29, wherein said administration comprises combining said extract with a foodstuff, and then orally administering said foodstuff.

36. The method of claim 29, wherein said hippocampal protein comprises an insulin receptor.

37. A method of inhibiting cholinesterase activity in the brain of a mammal comprising administering to said mammal a therapeutic amount of an extract of Liriopsis tuber.

38. The method of claim 37, wherein said extract of Liriopsis tuber is administered in an amount of from 0.1mg/kg to 500mg/kg.

39. The method of claim 38, wherein said extract is administered on a daily basis.

40. The method of claim 37, wherein said extract is administered via a route selected from the group consisting of oral administration, topical application, sterile injection, inhalation and rectal administration.

41. The method of claim 37, wherein said extract is concurrently administered with a pharmaceutically acceptable carrier, excipient or diluent.

42. The method of claim 37, wherein said administration comprises combining said extract with a beverage, and then orally administering said beverage.

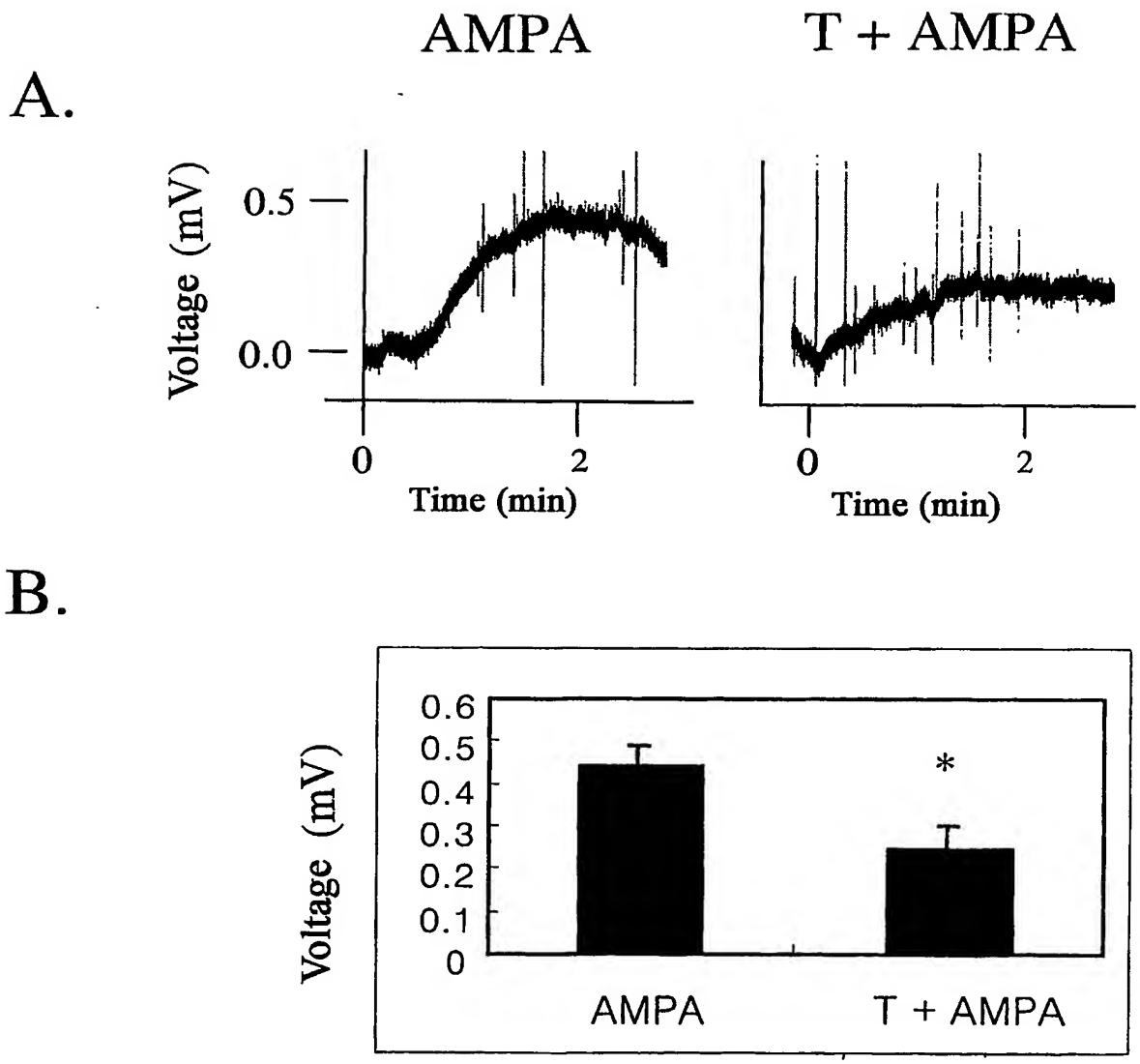
43. The method of claim 37, wherein said administration comprises combining said extract with a foodstuff, and then orally administering said foodstuff.

44. Use of an extract of Liriopsis tuber for the preparation of a medicament for preventing or treating neurodegenerative diseases.

45. Use of an extract of Liriopsis tuber for the preparation of a medicament for preventing or treating dementia.

46. Use of an extract of Liriopsis tuber for the preparation of a medicament for improving memory.

Fig. 1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0.3
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AMPA
T + AMPA

Fig.2

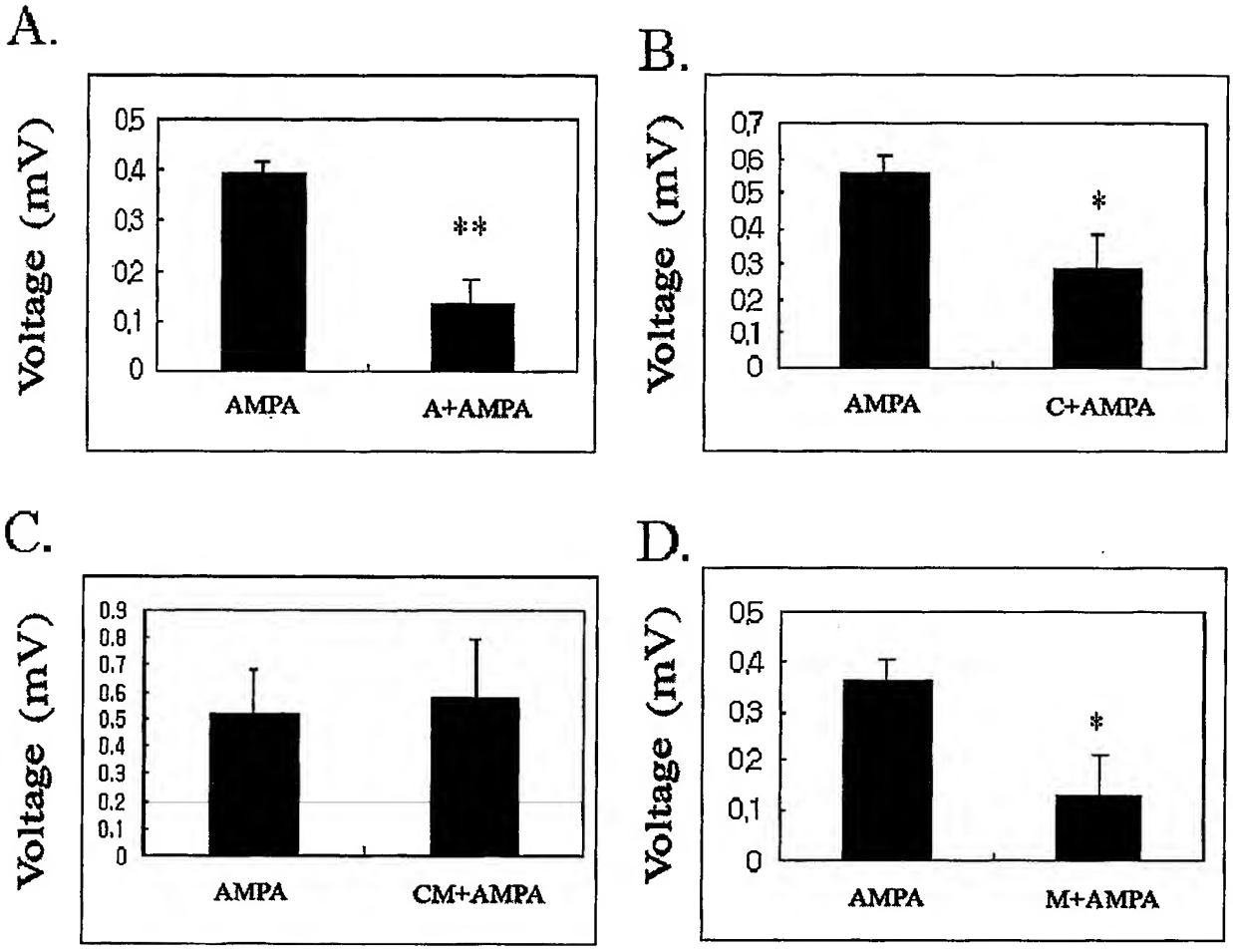
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Fig. 3

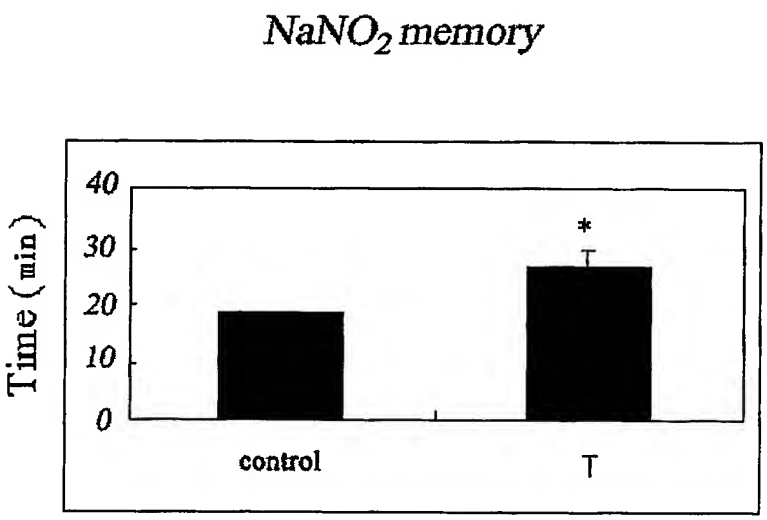
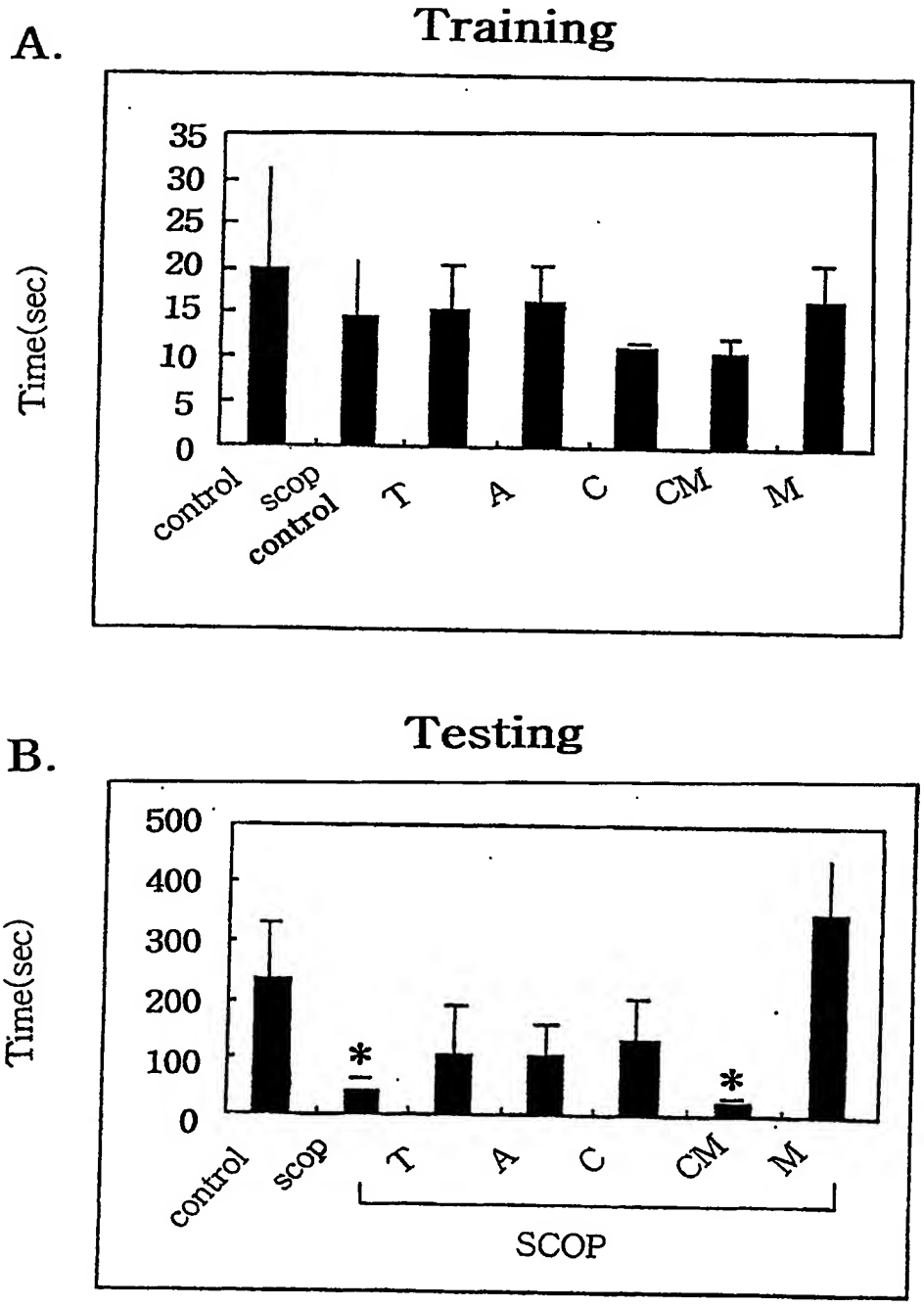


Fig. 4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B.
Testing
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C
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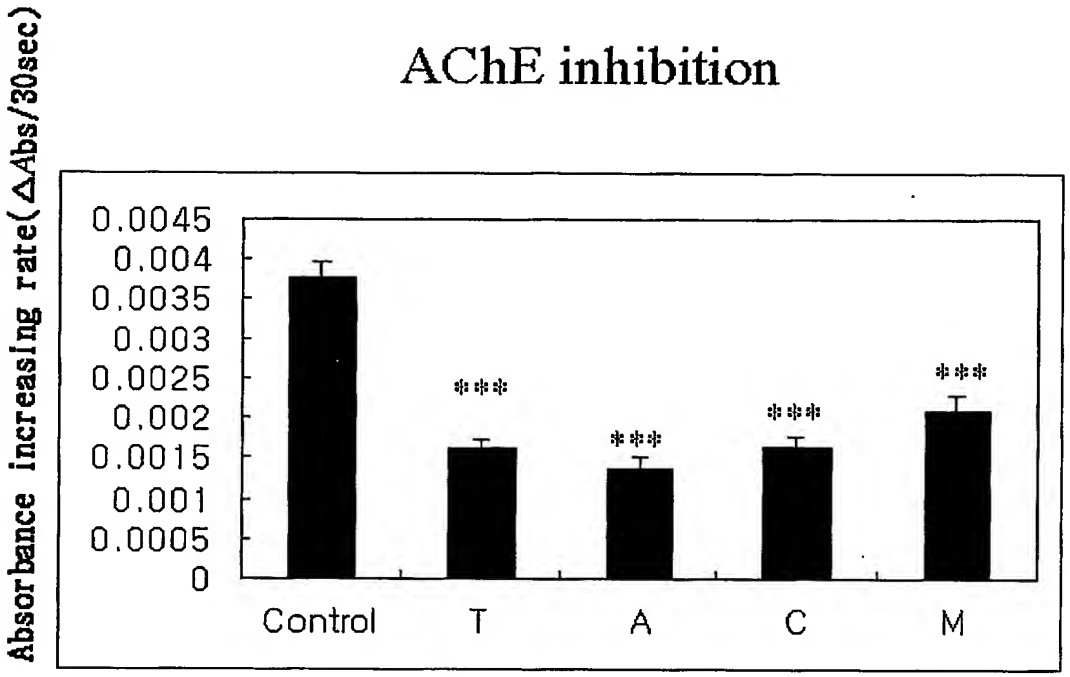
SCOP

Fig. 5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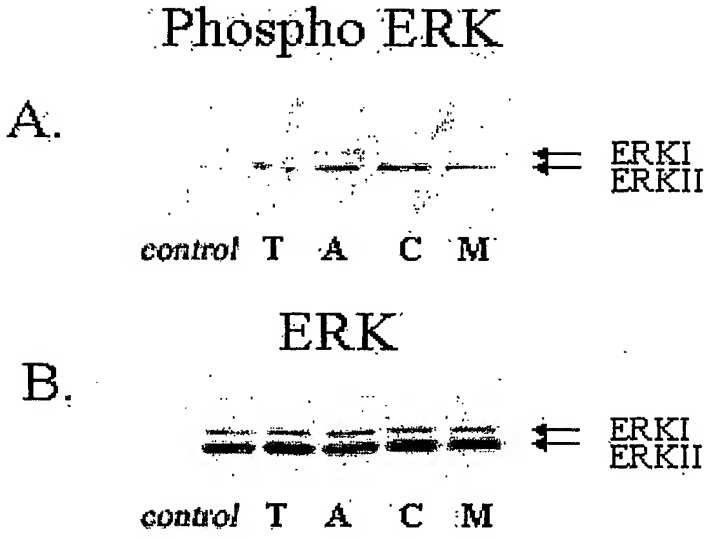
***
***
***
***
Control
T
A
C
M

Fig. 6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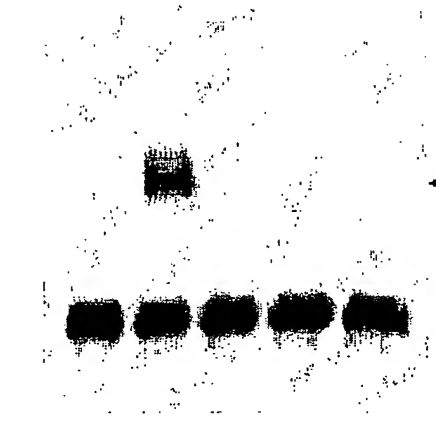
ERKII
control T A C M
ERK
B.
ERKI
ERKII
control T A C M

Fig. 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/00598

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 A61K 35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 35/78, A23L 2/38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
KOREAN PATENTS AND APPLICATIONS FOR INVENTIONS SINCE 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubMed on line

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, Y	KR 2003-0017069 A (Naturobiotech Co., Ltd.), 03 March 2003 See entire document	1-8, 15-36, 44-46
P, A	KR 2002-0025621 A (Lee, MH), 04 April 2002 See entire document	12-14
A	KR 2001-0110284 A (Lee, MH), 12 December 2001 See entire document	12-14
A	KR 2001-0073614 A (Yun, PJ), 01 August 2001 See entire document	12-14
A	Scemes E et al. 'Cholinergic mechanism in Liriope tetraphylla (Cnidaria, Hydrozoa)' In; Comp. Biochem. Physiol. C. 1986; 83(1): 171-8	37-43

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 JUNE 2003 (30.06.2003)	30 JUNE 2003 (30.06.2003)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	YEO, Ho Sup Telephone No. 82-42-481-5627

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR03/00598

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
KR 2003-0017069 A	03/03/2003	NONE	
KR 2002-0025621 A	04/04/2002	NONE	
KR 2001-0110284 A	12/12/2001	NONE	
KR 2001-0073614 A	01/08/2001	NONE	

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